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**Novel Pathways of Nitroaromatic Metabolism:  
Hydroxylamine Formation, Reactivity and Potential for  
Ring Fission for Destruction of TNT - CU1214**

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## **List of Acronyms**

2HA4ANT	2-Hydroxylamino-4-amino-6-nitrotoluene
2ADNT	2-Amino-4,6-dinitrotoluene
4HADNT	4-Hydroxylamino-2,6-dinitrotoluene
BLK	Bruhn, Lenke, and Knackmuss minimal medium
DHANT	2,4-Dihydroxylamino-6-nitrotoluene
DHANT	2,4-Dihydroxylamino-6-nitrotoluene
DNT	Dinitrotoluene
2,4-DNT	2,4-Dinitrotoluene
DoD	Department of Defense
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl- $\beta$ -D-1-thiogalactopyranoside
LB	Luria-Bertani Broth
NB	Nitrobenzene
NOM	Natural Organic Matter
SPE	Solid Phase Extraction
TAT	2,4,6-Triaminotoluene
TNT	2,4,6-Trinitrotoluene
UV	Ultraviolet
YM	Yellow Metabolite



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## Executive Summary

Contamination by 2,4,6-trinitrotoluene (TNT) is widespread at many sites where explosives have been manufactured and stored. TNT contamination occurs in the environment predominantly in surface and shallow subsurface soils. The presence of this contamination in easily accessible surficial soils makes it amenable to treatment by either *in situ* or *ex situ* engineered systems. Bioremediation has come into favor as the treatment of choice for munitions contamination because of the prohibitively high cost of the treatment alternative, incineration. To this end, this research was designed to provide information required for development of bioremediation systems to treat TNT contamination. This research investigated biological transformation of TNT with the primary goal of furthering the understanding of the fundamental biochemical mechanisms responsible for transformation of TNT and its fate in the environment. This research explored the products of novel TNT transformation pathways and determined the mechanisms of TNT transformation and identified the enzymes responsible. The fate of TNT transformation products in natural systems was investigated along with strategies to direct TNT metabolism to ring fission products and mineralization of TNT. Studies were primarily focused on transformation of TNT by the anaerobic organism *Clostridia acetobutylicum* and the aerobic organism *Pseudomonas pseudoalcaligenes* strain JS45.

The reactivity of partially reduced metabolites of 2,4,6-trinitrotoluene (TNT) produced by *C. acetobutylicum*, namely arylhydroxylamines and nitrosoarenes, was evaluated with a simple biological system and with components of soil natural organic matter (NOM). In the simple bioreduction system of *Clostridium acetobutylicum* cell-

free extract/molecular hydrogen (electron donor), 10% of the initial  $^{14}\text{C}$  was found bound to solid proteinaceous material following sequential anaerobic/aerobic treatment. A review of the nitroso and hydroxylamino functional group chemistry revealed that the nitroso-thiol reaction was most likely responsible for the reaction with proteins. The introduction of a model thiol, 1-thioglycerol, into an anaerobic mixture of 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 2,4-dihydroxylamino-6-nitrotoluene (DHANT) resulted in the formation of a new product, only when the reaction mixture was exposed to air. The reactivity of arylhydroxylamines and nitrosoarenes with standard humic acids was investigated using 4HADNT and nitrosobenzene as model compounds, respectively. Contrary to results reported by others, 4HADNT was found to be nonreactive towards humic acid at humic acid concentrations in excess of dissolved organic matter concentrations found in nature. Conversely, nitrosobenzene reacted rapidly with humic acids, with the extent of reaction being highest for humic acids that had a high protein content. Humic acids that were pretreated with a thiol derivatizing agent showed diminished capacity for reaction with nitrosobenzene. Since nitroso intermediates from TNT reduction are difficult to synthesize and are rarely observed in nature due to their high instability, their electrophilic characteristics were evaluated using a molecular modeling approach. Molecular models of potential TNT nitroso intermediates were compared with those of the strongly electrophilic nitrosobenzene. The comparison revealed that 2-nitroso-4-hydroxylamino-6-nitrotoluene was more likely to react similarly to nitrosobenzene than to 4-nitroso-2,6-dinitrotoluene.

Studies which investigated the enzymatic mechanism responsible for transformation of TNT by *C. acetobutylicum* were focused on determination of whether

the Fe-only hydrogenase is the primary enzyme in *C. acetobutylicum* responsible for reduction of TNT. Using purified enzyme, it was determined that the hydrogenase is capable of reducing TNT in an H<sub>2</sub> atmosphere in the absence of alternate electron donors, which subsequently allowed the kinetic parameters to be measured. Further studies were carried out to examine a causative relationship between the activity of hydrogenase present in a cell system and the corresponding rates of TNT reductase activity. The results of the studies demonstrated that the hydrogenase enzyme proposed is responsible for the major nitroreductive capability of *C. acetobutylicum*.

Further studies with *C. acetobutylicum* investigated the potential for mineralization of TNT using a two-stage anaerobic-aerobic incubation. We report that CO<sub>2</sub> production (confirmed using <sup>14</sup>C-TNT) occurred during a two-stage microbial incubation as a result of a combined biological-chemical process. <sup>14</sup>C-TNT was transformed by actively growing *C. acetobutylicum* and subsequently exposed to active aerobic cultures known to metabolically degrade 2,4-DNT. Aerobically-maintained reactors with unrestricted growth converted 13.3 to 14.1% of the initial radiolabeled metabolite to <sup>14</sup>CO<sub>2</sub>. Active reactors produced significantly more <sup>14</sup>CO<sub>2</sub> than aerobic assays in which growth was restricted (via autoclaving, mineral omission, or biocide amendments). Production of <sup>14</sup>CO<sub>2</sub> in these restricted-growth reactors ranged from 7.9 to 9.7%, suggesting that an abiotic pathway was responsible for greater than 56% of the observed mineralization yield. It was determined that incubation under anaerobic conditions in the second stage of the dual-step process limited mineralization (1.1 to 1.7% <sup>14</sup>CO<sub>2</sub>) by blocking growth and/or auto-oxidation pathways, while the addition of structurally similar compounds to TNT did not lead to mineralization. It is postulated

that the clostridial pathway that yields hydroxylated intermediates is key to providing more suitable starting compounds for initiation of the mineralization process.

We also investigated the mechanism and enzymes responsible for transformation of TNT by *P. pseudoalcaligenes* JS45, a soil organism isolated for its ability to grow on nitrobenzene under aerobic conditions. Cells grown on nitrobenzene were previously reported to transform TNT by partial reduction of the nitro groups catalyzed by nitrobenzene nitroreductase. The nitroreductase produces only hydroxylamino derivatives of TNT and the final product is an unidentified yellow metabolite. Here we discovered that *P. pseudoalcaligenes* JS45 contains multiple nitroreductases, including a unique constitutively expressed nitroreductase that converts TNT to 2HADNT. A wide range of bacteria, including *E. coli*, can catalyze the conversion of TNT to amino derivatives. All of the nitroreductases that have been purified, however, seem to catalyze only the reduction to the hydroxylamino derivatives. It has been a mystery how the conversion of the hydroxylamino compounds to the amines is carried out. In JS45 we have clearly demonstrated the activity of the enzyme that produces amino compounds from TNT. The existence of the enzyme is crucial to avoid accumulation of the much more reactive hydroxylamino compounds. Purification and characterization of the novel enzyme will reveal whether it is widespread and whether its activity is responsible for the often observed accumulation of monoamino derivatives of TNT under aerobic conditions. The advances in understanding from JS45 will allow prediction and enhancement of the activity in a variety of systems.



2ADNT is further transformed by the action of the same reductase, but the product of the reaction has not been isolated. The currently available evidence indicates that 2-amino-4-hydroxylaminonitrotoluene is formed and subsequently converted by the mutase enzyme to the corresponding aminophenol which accumulates transiently and then disappears. When the experiments were carried out with  $^{14}\text{C}$ -TNT the products eventually became unextractable and bound to soil when it was included in the reaction mixture.

Enzymes of the nitrobenzene degradation pathway that effect transformation of TNT and its metabolites were also identified, and experiments with purified enzymes demonstrated that a mutase enzyme converts DHANT to the same yellow metabolite that is produced by cells grown on succinate. Experiments with  $^{14}\text{C}$ -TNT demonstrated that JS45 can mineralize TNT when the nitrobenzene degradation pathway is expressed, but mineralization is suppressed by the presence of soil because of binding of polar metabolites of TNT or their precursors by soil components.

Nitrobenzene is more acutely toxic than TNT and thus is unlikely to be used as a primary growth substrate in TNT remediation systems. However, this project has shown that constitutive enzymes, including the nonspecific nitroreductase(s) and the constitutively expressed HabA will transform TNT almost as well as the fully expressed enzymes of the nitrobenzene-degradation pathway. *P. pseudoalcaligenes* JS45 is a natural environmental isolate and therefore faces few regulatory restrictions on its use in remediation systems. With the idea that the constitutively expressed enzymes of JS45 could be useful in TNT remediation systems *in situ*, we examined alternative carbon sources for cometabolism of TNT by JS45.

Tests with glucose, succinate, and molasses revealed that the best growth and TNT transformation resulted from molasses as carbon source. The molasses-grown cells took less than 24 hours to transform 100  $\mu$ M TNT. Molasses is a cheap and readily available carbon source with demonstrated value for cometabolism. The difference between the system described here and previously described systems using molasses as electron donor is that the final product is not a polyamino compound due to the action of the mutase enzyme in JS45. Remediation systems based on cometabolism of TNT by JS45 during growth on molasses could be investigated for field application where the bulking of the contaminated material inherent in composting is to be avoided.

## **Objective**

The goals of the studies were to examine the biochemical mechanism of TNT transformation in novel degradation pathways, and to use this fundamental information to develop strategies that harness the activity in remediation systems.

The specific objectives of the research were to:

1. Identify the products of novel TNT transformation pathways
2. Determine the mechanism of TNT transformation and identify the enzymes responsible. Characterize the properties of the enzymes and their regulation.
3. Examine the fate of TNT transformation products in natural systems.
4. Develop strategies to direct TNT metabolism to ring fission products.
5. Examine the potential for mineralization of novel TNT metabolites.

## Background

Nitroaromatic compounds are widespread contaminants at DOD facilities. Over 700,000 cubic yards of soil and 10 billion gallons of groundwater require treatment (SERDP, 1993). The cost to complete cleanup of Military Munitions Response Program sites is estimated to be \$18.7 billion through 2010 (DEP, 2004). TNT is the primary contaminant at these sites, along with dinitrotoluenes (DNT), and the other nitro substituted explosives (i.e., RDX and HMX). Current approaches used for site remediation typically involve excavation of contaminated soils, followed by incineration or composting, and pump-and-treat for contaminated groundwater.

The development of *in situ* bioremediation processes for the treatment of TNT and other nitroaromatics would greatly improve DOD's ability to restore contaminated sites in a more cost-effective manner (Anderson et al., 1999). The factor that has limited the development of *in situ* bioremediation processes for treatment of TNT contaminated soils or groundwater is the inability of bacteria to use TNT as a growth substrate.

Characteristics common to nitroaromatic compounds (i.e., electron deficient pi-orbitals and high redox potentials) can reduce the ability of oxygenase enzymes to catalyze an initial electrophilic attack on the aromatic ring and can cause reductive metabolic pathways to become favorable. Aryl nitro groups are labile and can undergo reduction reactions catalyzed by a range of electron transfer proteins found in microorganisms. In general, the propensity for reduction increases with the degree of nitro-substitution. For example, aerobic bacteria can use initial oxidative metabolism to metabolize 2,4-dinitrotoluene and 2,6-dinitrotoluene, but reductive metabolism is predominant with TNT.

Reduction of the nitro groups makes the molecule more resistant to subsequent reduction and more susceptible to oxidation. For this reason, current *ex situ* treatment systems (i.e., composting and slurry reactors) have focused on co-metabolic transformations that lead to binding (also referred to as sequestration or humification) of metabolites to soil and amendments. This approach to TNT bioremediation does not result in ring fission, and because the products are difficult to characterize or monitor, the treatment endpoint remains controversial. The application of similar techniques for *in situ* treatment is unlikely as these processes use overwhelming amounts of co-substrate in heavily engineered systems to maintain very low redox potentials for extended periods. More recently, it has been suggested (Lenke et al., 2000) that partial reduction under anaerobic conditions can lead to binding and humification during subsequent aerobic treatment. The amount of carbon source and electron donors required are still daunting and the treatment does not result in destruction of the aromatic ring. Therefore, alternate technologies which alter the base molecular structure of TNT (i.e., loss of aromaticity, mineralization) are required.

Multiple organisms share intermediates in TNT transformation pathways that could lead to further transformation products which no longer display aromatic characteristics (Figure 1). The central metabolite in the pathways of *C. acetobutylicum* and *P. pseudoalcaligenes* JS45 is 2,4-dihydroxylamino-6-nitrotoluene (Fiorella and Spain, 1997), which is hydroxylated via rearrangement (Hughes et al., 1998) and then degraded to polar products. In the systems we have studied, the rearrangement of DHANT serves as a gateway to a novel pathway for TNT transformation.

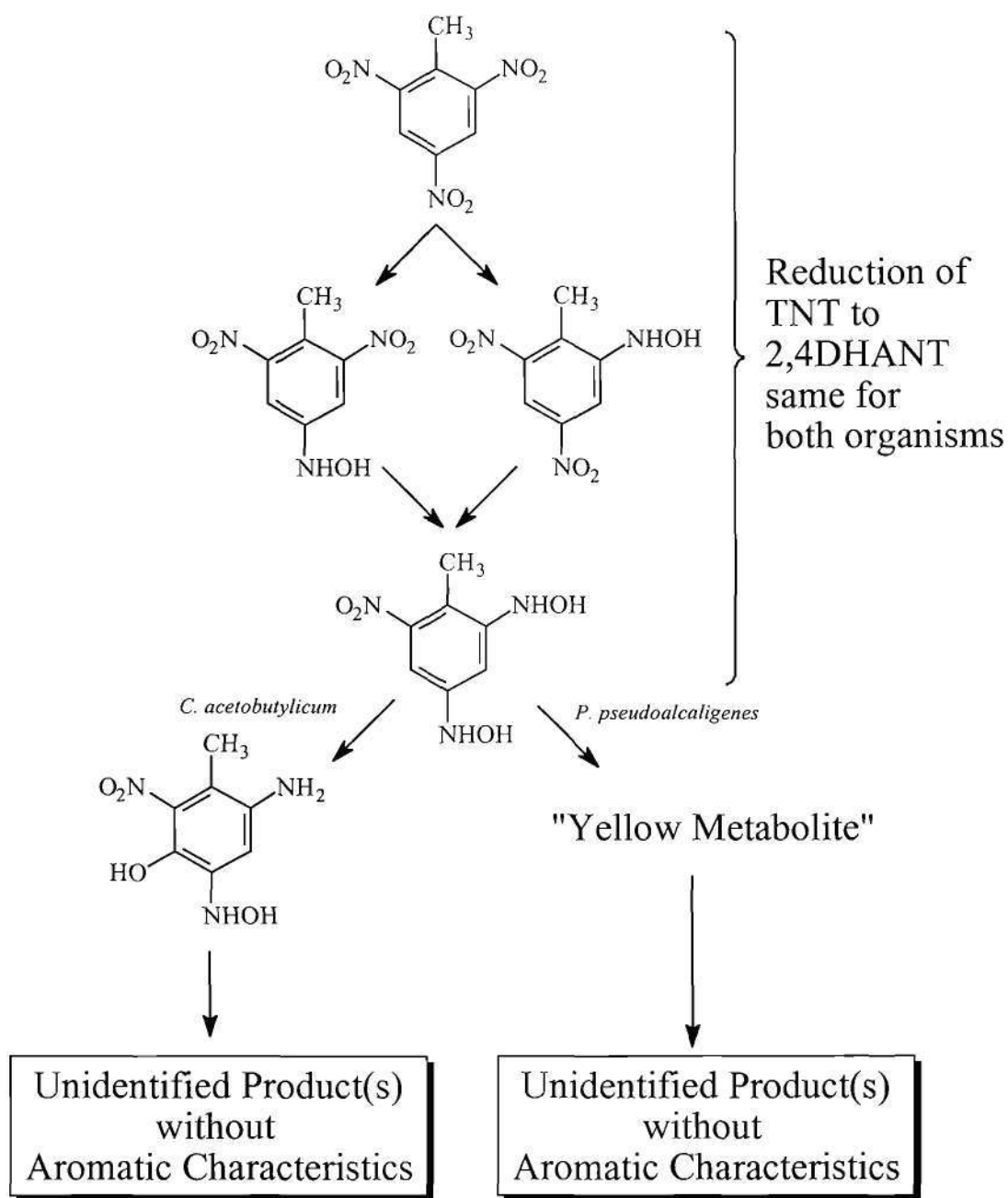


Figure 1. Novel pathway of TNT transformation through 2,4-dihydroxylamino-6-nitrotoluene (DHANT) for *C. acetobutylicum* (Hughes et al., 1998) and *P. pseudoalcaligenes* (Fiorella and Spain, 1997) that results in products that do not display aromatic characteristics (no UV absorbance, not extractable with organic solvent, highly polar). The "yellow metabolite" is believed to be a rearrangement product similar to that detected with *C. acetobutylicum*.

Aerobic Biodegradation. In recent years, it has been demonstrated that a number of nitroaromatic compounds are susceptible to aerobic microbial degradation and a considerable body of knowledge has been developed about the catabolic pathways of nitroaromatic metabolism during aerobic biodegradation (Nishino, Spain *et al.* 2000; Nishino and Spain 2004). Of particular interest in these studies are the pathways used by aerobic bacteria to remove the nitro group during conversion of the nitroaromatic compounds to central metabolites. The nitro group can be released as nitrite during aerobic microbial metabolism by three distinct mechanisms: a) dioxygenation of the aromatic ring to a dihydroxy intermediate (Spanggord, Spain *et al.* 1991; Ecker, Widmann *et al.* 1992; Nishino and Spain 1992; Haigler, Wallace *et al.* 1994; Nadeau and Spain 1995; Nishino and Spain 1995; Nishino, Paoli *et al.* 2000); b) monooxygenation to an epoxide (Zeyer and Kearney 1984; Spain and Gibson 1991; Schäfer, Harms *et al.* 1996); or, c) hydride-Meisenheimer complex formation (Lenke and Knackmuss 1992; Lenke and Knackmuss 1996; Behrend and Heesche-Wagner 1999; Rieger, Sinnwell *et al.* 1999). Alternatively, the nitro group can be released in the form of ammonia when pathways proceed via initial reduction and formation of an aryl hydroxylamine. (To date there is little evidence for an aerobic pathway that involves complete reduction to the amine prior to ring fission by the same bacterium). Because direct oxygenase attack on TNT had not been observed until recently (Tront and Hughes, 2005), and since it is uncertain whether productive TNT metabolism can occur from TNT hydride-Meisenheimer complexes (Vorbeck, Lenke *et al.* 1994; Haïdour and Ramos 1996; French, Nicklin *et al.* 1998; Vorbeck, Lenke *et al.* 1998; Rieger, Sinnwell *et al.* 1999; Pak, Knoke

*et al.* 2000; Weiß, Geyer *et al.* 2004), pathways involving initial reduction to hydroxylamines were of particular interest in this project.

The elimination of aryl nitro groups as ammonia via hydroxylamino-compounds occurs via two distinct mechanisms. In one mechanism, the hydroxylamino-compound is attacked by an enzyme described as a hydroxylaminolyase (Groenewegen and de Bont 1992), which produces a corresponding catechol and eliminates ammonia. Although details of the reaction mechanism itself remain unclear it is known to be involved in the metabolism of 4-nitrotoluene (Haigler and Spain 1993; Rhys-Williams, Taylor *et al.* 1993), 4-nitrobenzoic acid (Groenewegen, Breeuwer *et al.* 1992; Yabannavar and Zylstra 1995), and 3-nitrophenol (Meulenberg, Pepi *et al.* 1996). The second pathway that includes a hydroxylamino-intermediate (depicted in Figure 2) involves a mutase catalyzed intramolecular rearrangement of the hydroxylamino-compound to an *o*-aminophenol. The *o*-aminophenols serve as *meta*-ring cleavage substrates for highly specific dioxygenases, and the reactions result in the formation of aminomuconic semialdehydes. The amino group is released as ammonia in subsequent reactions. Nitrobenzene (Nishino and Spain 1993), 2-chloro-5-nitrophenol (Schenzle, Lenke *et al.* 1999), 4-chloronitrobenzene (Katsivela, Wray *et al.* 1999), 4-nitrotoluene (Spiess, Desiere *et al.* 1998) as well as 3-nitrophenol (Schenzle, Lenke *et al.* 1997; Schenzle, Lenke *et al.* 1999) are degraded by the mutase mediated pathway via *o*-aminophenols.

Interestingly, partial reduction of TNT to hydroxylamino-dinitrotoluenes and amino-dinitrotoluenes takes place prior to oxidation during its metabolism by fungi (Fritsche, Scheibner *et al.* 2000). As in other systems, initial reduction results in



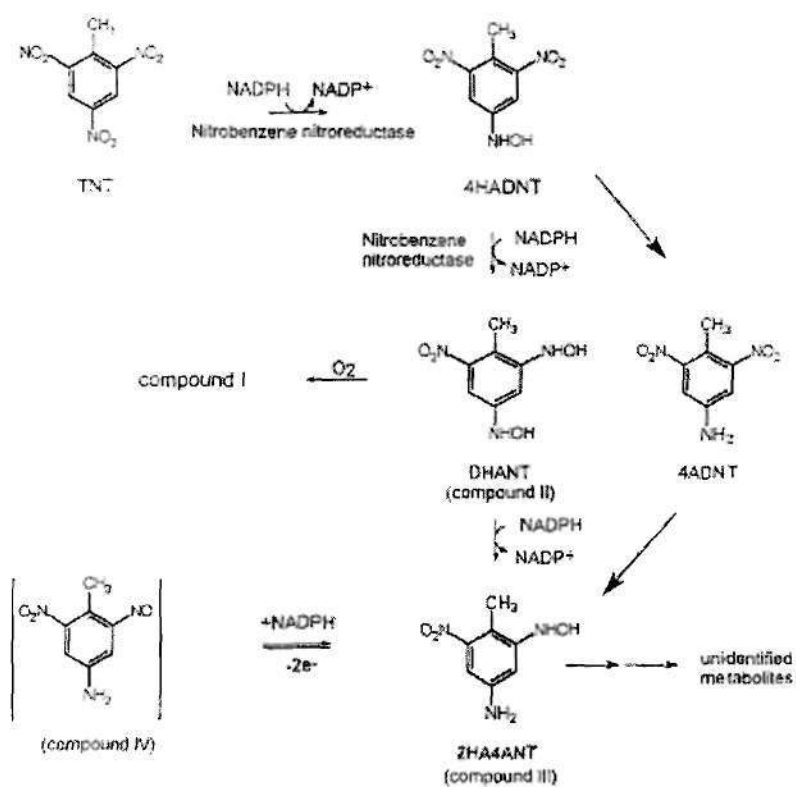


Figure 2. Proposed pathway for TNT transformation by nitrobenzene-grown cultures of *P. pseudoalcaligenes* (Fiorella and Spain 1997).

compounds that are more prone to oxidative attack. Evidence of analogous forms of TNT metabolism in bacterial culture has been reported, although little mechanistic information exists (Alvarez, Kitts *et al.* 1995).

*Pseudomonas pseudoalcaligenes* strains JS52 and JS45 grow aerobically on nitrobenzene using an initial reductive pathway (Figure 3) (Nishino and Spain 1993; He and Spain 1999). In this case, nitrobenzene nitroreductase converts nitrobenzene to hydroxylaminobenzene, which is then converted to 2-aminophenol by the action of hydroxylaminobenzene mutase. The 2-aminophenol is then subject to oxidative attack and ring fission. The mutase does not require cofactors and catalyzes an intramolecular rearrangement of the molecule (He, Nadeau *et al.* 2000). The reaction leads to the substitution of a hydroxyl group on the aromatic ring without the action of an oxygenase and without the addition of water. 2-Aminophenol is the substrate for a novel ring-fission dioxygenase that catalyzes the opening of the aromatic ring and the formation of aminomuconic semialdehyde. Several other strains that use analogous pathways for the degradation of 3-nitrophenol (Schenzle, Lenke *et al.* 1997), 4-nitrotoluene (Spiess, Desiere *et al.* 1998), and chloronitrobenzene (Katsivela, Wray *et al.* 1999) have been discovered in the past few years. The results described above suggest that the enzymes used for the reductive conversion of nitroaromatic compounds to ring-fission substrates are widespread. They may have different substrate preferences, but the mechanisms are probably the same.

These results have since led to the discovery that the nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* can catalyze the transformation of TNT to 4-hydroxylamino-2,6-dinitrotoluene and then to 2,4-dihydroxylamino-6-nitrotoluene

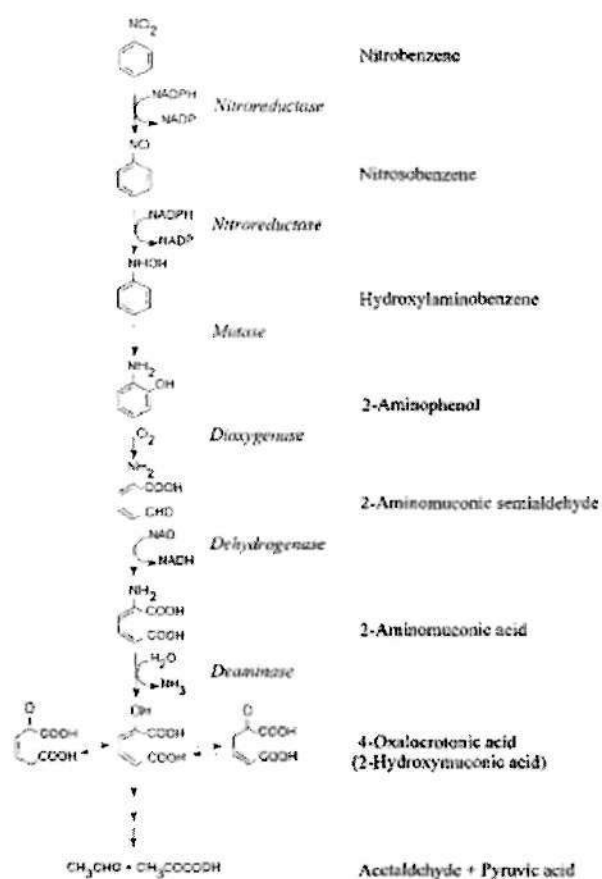


Figure 3. Partially reductive pathway for degradation of nitrobenzene (modified from (Nishino and Spain 1993; He and Spain 1999)).

(DHANT) (Fiorella and Spain 1997). DHANT is also a central intermediate in the metabolism of TNT by clostridia. The nitrobenzene nitroreductase does not transform DHANT, but other reductases in crude cell extracts catalyze the slow reduction of DHANT to 2-hydroxylamino-4-amino-6-nitrotoluene (2HA4ANT). Both DHANT and 2HA4ANT are transformed to more polar metabolites by enzymes in crude cell extracts. The transformations require oxygen, which suggests that the reactions involve incorporation of molecular oxygen. DHANT is also converted to a polar yellow metabolite (YM) that cannot be extracted from the aqueous phase with organic solvents. It is sensitive to acid and base and the UV/vis spectrum reveals maxima at 258, 325, and 405 nm. The mass spectrum gave a molecular ion of 183. The properties of the molecule are consistent with the structure of a toluene ring with two amino groups, a hydroxyl group, and a nitro group. Such a compound could be formed by a mutase-catalyzed rearrangement of DHANT in a reaction similar to the conversion of hydroxylaminobenzene to 2-aminophenol. The fact that the conversion of DHANT to the yellow metabolite requires air suggests on the other hand that an oxygenase is involved in the reaction. An alternative explanation would be a nonenzymatic oxidation of one of the functional groups of the molecule. Enzymes in cell extracts further metabolize the YM and nitrite is released during the process. It is not clear whether nitrite is released during the formation or the subsequent metabolism of the yellow metabolite. In any case, the YM and 2HA4ANT seem to be the gateway compounds to the subsequent conversion of the TNT metabolites to molecules that have lost their aromatic character and are no longer recognizable as TNT derivatives. Such compounds can bind to humic material or could be further degraded. Such binding and conversion to non-identifiable polar

products can cause the TNT related toxicity to disappear completely (Lenke, Achtnich *et al.* 2000). Alternatively, it may be possible to engineer pathways for the mineralization of the extensively modified TNT metabolites once they are identified.

Reductive Cometabolism. Fortuitous reduction of the aryl nitro groups of poly-nitroaromatics is commonly observed in cultures of aerobic and anaerobic bacteria (Ramos, Caballero *et al.* 2004). Reductive pathways proceed through two intermediate forms before the process is complete, thus the reduction of TNT (or other poly-nitroaromatic compounds), has the potential to yield a variety of intermediates and products. Specifically, the groups formed during nitro group reduction include the nitroso-group (R-NO), the hydroxylamino-group (R-NHOH), and the amine (R-NH<sub>2</sub>). Nitroso-groups are rarely observed in microbial metabolism, because further reduction to hydroxylamines is strongly favored. Complete reduction to the amine can occur, although some organisms appear to be deficient in this ability and the accumulation of hydroxylamines has been observed frequently even under strong reducing conditions. Most of the nitroreductases studied to date catalyze the conversion of the nitro group to the hydroxylamine (Somerville, Nishino *et al.* 1995; Koder, Haynes *et al.* 2002). The enzymes responsible for the subsequent reduction to the amine are a mystery.

Reductive reactions can be involved in the complete degradation of nitroaromatic compounds by aerobic bacteria. However, cometabolic reduction processes appear to be a more widely distributed form of reductive metabolism – particularly with TNT (Rieger and Knackmuss, 1995). The rate and extent of the cometabolic reduction of nitroaromatic compounds is strongly influenced by other substituents on the ring in

addition to the nitro group(s) (Haderlein and Schwarzenbach, 1995). For example, the electron withdrawing characteristics of two additional nitro groups facilitates the initial *reduction of one nitro group of TNT. The presence of ring activating groups (amines, hydroxyl groups, etc.)* will produce the opposite effect. For this reason, cometabolic TNT reduction under aerobic conditions rarely proceeds beyond the reduction of a single nitro group. The stronger reducing conditions present under anaerobic conditions may lead to the reduction of two or more nitro groups of TNT.

The ability to reduce nitroaromatic compounds is also related to organism-specific properties including the expression of low-redox electron transfer proteins that exhibit nitroreductase activity, and the rate of electron transfer within the organism. Based upon these two organism-specific factors, considerable interest has been focused on the propensity of fermentative bacteria to reduce nitroaromatic compounds (Ederer et al., 1997). Clostridia, in particular, rapidly reduce aryl nitro groups due to the high expression by clostridia of low redox Fe-S electron transfer proteins, and the need to recycle NADH rapidly during anaerobic glycolysis (i.e., The Pasteur effect). Interestingly, the products of fermentative TNT reduction appear to be predominantly hydroxylamino-derivatives that undergo rearrangement to substituted aminophenols (Hughes et al., 1998; Hughes et al., 1998b; Hughes et al., 1999). This rearrangement reaction is similar to those discussed in the previous section, although it appears that differences in the mechanism involved may exist (*para*-rearrangement instead of *ortho*-rearrangement is observed).

Research focused on the role of hydroxylamines in the rapid anaerobic metabolism of TNT, 2,4-DNT, and 2,6-DNT by clostridia has demonstrated that

dihydroxylamino-forms of each nitroaromatic were central intermediates of metabolism. In the case of TNT, further transformation yielded an aminophenol product via a Bamberger rearrangement (Hughes et al., 1998) as depicted in Figure 1. Subsequent studies have concluded that the reduction of the nitro group is due to a Fe-S containing hydrogenase, which also appears to catalyze rearrangement and hydroxylation. The Fe-S clusters present in this hydrogenase is common to a number of enzymes in clostridia and other bacteria. Carbon monoxide dehydrogenase from *C. thermoaceticum* containing identical Fe-S clusters also transformed TNT to the same aminophenol product (Huang et al., 1999). While the formation of hydroxylamino groups was observed in cell cultures and purified enzyme systems for all nitroaromatics tested, the formation of aminophenols was only observed from TNT. Whether this is due to steric effects (i.e., the shape of the molecules involved) or electronic effects (i.e., the electron withdrawing characteristics of substituent groups) is not known. The final products of TNT and DNT transformation via hydroxylamine pathways were highly oxygen sensitive (Wang et al., 2000) and products of decomposition do not exhibit aromatic characteristics. In particular, they do not have a UV absorbance in the aromatic region, can not be extracted into organic solvent, display characteristics of a zwitterion (expected for an aminated organic acid), and are highly polar. The long term fate, toxicity, and potential for mineralization of these products have not yet been investigated. Ames tests have confirmed that these products are not mutagenic (Padma et al., 1999).

An interesting component to these studies is the rapid nature of transformation observed in the early growth phase of the organism. After the organism's metabolism

switches from acidogenic (fermentation to acetic acid and butyric acid) to solventogenic (fermentation to ethanol and butanol), the ability to catalyze TNT transformation disappears (Khan et al., 1997). We have demonstrated that this loss of activity results from strict metabolic control of hydrogenase activity through testing of mutant strains deficient in the genes required for solventogenic growth and with selective inhibitors of the hydrogenase. These results imply that it will be important to understand the regulation of pathway expression to maintain desired activity *in situ*.

The product of complete TNT reduction is 2,4,6-triaminotoluene (TAT). TAT has been postulated as a central intermediate in the anaerobic treatment of TNT (Crawford, 1995), even when it has not been detected. It has been demonstrated that reduction under iron-reducing conditions leads to rapid production of TAT (Heijman et al., 1995). The amino groups of TAT are strong ring activators, making the ring susceptible to electrophilic attack. As reviewed by Lenke, et al., (2000) TAT is readily oxidized by oxygen in abiotic reactions catalyzed by metal ions such as  $Mn^{2+}$ . The products of this process appear to be polymers that are at best difficult for bacteria to degrade. Studies have also shown that TAT will “disappear” in anaerobic systems, possibly forming tetraaminoazobenzenes or polynuclear azo compounds. Thus it does not appear that the complete reduction of TNT to TAT will result in the formation of ring fission precursors that are amenable to complete destruction. Conversion to TAT and subsequent humification might be an acceptable strategy for treatment of excavated soil contaminated with TNT in anaerobic reactors where high concentrations of additional carbon sources can be added and low redox potentials can be maintained.



Anaerobic-Aerobic Treatment of TNT. Because the initial metabolism of TNT involves reduction, and reduced products should be more amenable to oxygenase attack, two-stage treatment of TNT contaminated soils has been investigated. There are several process configurations that achieve this two-stage approach, including composting (Breitung et al., 1996; Bruns-Nagel et al., 1998), slurry reactors (Funk et al., 1993; Manning et al., 1996; Greist et al., 1998), and in some cases natural attenuation. Regardless of the system employed, anaerobic-aerobic treatment appears to yield a high degree of bound residues and does not result in destruction of the aromatic ring (Lenke et al., 2000). Because the products are difficult to characterize or monitor, and because the process is only useful for excavated soil, the effectiveness of anaerobic-aerobic treatment processes remains controversial. The process of binding is an area of continued study (Lenke et al., 2000), but it appears to result from reactions of partially reduced intermediates with the organic matrix of soil or compost amendments (Daun et al., 1998; Lenke et al., 1998). Despite the strong reducing conditions present, the rate of binding reactions in these processes appears to be slow, relative to the rate of TNT transformation and it should be possible to route product distribution away from bound residues in *in situ* processes. It is our hypothesis that this could best be achieved for TNT through pathways of initial reductive metabolism that yield the rapid formation of aerobic ring fission precursors (i.e., hydroxylated rearrangement products). Such strategies involving reduction only to the hydroxylamine would require far less carbon addition and less dramatic shifts in redox potential and so would be considerably less energy intensive. The properties of such a system would be much more amenable for *in situ* treatment of

contaminated soil and groundwater using optimization of “amendments” to induce desired pathways, bioaugmentation, or through metabolic engineering.

## Materials and Methods

An expansion of materials used and experimental methodology for work completed with *C. acetobutylicum* are described with data in Appendix A. The methodology used for experimentation with *P. pseudoalcaligenes* JS45 is described here.

**Bacteria and culture methods.** *P. pseudoalcaligenes* JS45 was grown in a nitrogen free minimal medium (BLK) (Bruhn, Lenke *et al.* 1987) with NB provided as the sole carbon, nitrogen and energy source (Nishino and Spain 1993). To grow high density cultures ( $1 < A_{600} < 11$ ), the amount of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in BLK was increased 10-fold. The strain was maintained on BLK agar plates provided with NB vapor as previously described (Nishino and Spain 1993). Small shake flask cultures (100 ml) were inoculated with JS45 and provided 2.5 mM nitrobenzene, and grown overnight at 30 °C, with shaking at 200 rpm. Shake flask cultures were used to inoculate a 2 L (1.3 L working volume) bioreactor (New Brunswick Scientific, Edison, NJ). NB was fed to the bioreactor via a syringe pump. The NB flow rate was high enough to support cell growth, but low enough so that there was no detectable nitrobenzene in the culture medium by high performance liquid chromatography (HPLC) analysis. Cultures were monitored for growth by measuring  $A_{600}$  on a Cary 3E spectrophotometer.

**Transformation of TNT.** TNT (100 mM) dissolved in HPLC grade methanol, was added to NB-grown cultures of JS45 either in shake flasks or in the 2 L bioreactor to give a final TNT concentration of 200 to 1000  $\mu\text{M}$ . Disappearance of NB and TNT and appearance of metabolites were monitored by HPLC.

**Analytical methods.** HPLC was performed on a Hewlett Packard HP-1090 system equipped with a diode array detector. Analyses were performed with a Spherisorb C8 column as previously described (Fiorella and Spain 1997) or with a Merck Chromolith Performance RP-18e column (100 mm x 4.6 mm I.D.) using a combined flow and solvent gradient. The initial flow rate was 0.75 ml/min and the mobile phase consisted of a 95:5 ratio of part A (13.5 mM trifluoroacetic acid in water) and part B (6.75 mM trifluoroacetic acid in acetonitrile). At 3.01 min the flow was stepped up to 1 ml/min and the mobile phase changed to 65:35 part A:part B and the flow rate was increased in a linear gradient from 1.0 to 4.0 ml/min over 4 min then held at 4.0 ml/min for 0.5 min. NB, TNT, and metabolites were monitored at  $A_{254}$  and the yellow metabolite at  $A_{420}$ .

**Metabolite purification.** Culture fluids were clarified by centrifugation at 25000 x g then pumped through Bond Elut C-18 solid phase extraction (SPE) cartridges (Varian Inc.). The fluids that passed through the column and material extracted from the column with deionized water, which included the YM, were collected and extracted onto an Envi-Carb graphitized nonporous carbon (Supelco) SPE column. The cartridge was prepared and extracted according to the manufacturer's method for extraction of base-neutral and acidic pesticides (Supelco 1997). The YM and one other compound eluted in the acidic fraction. The solvents were removed by flash evaporation. Material that remained bound to the C-18 column was sequentially eluted with 30%, 50% and 70% methanol in deionized water. The methanol was removed from the fractions by flash evaporation and the aqueous phase was extracted with ethyl acetate, which was in turn removed by flash evaporation to concentrate the metabolites.

**Preparation of DHANT.** DHANT was produced by incubating partially purified nitrobenzene nitroreductase in phosphate buffer with TNT (200  $\mu$ M) and NADPH (500  $\mu$ M) for 1 h at room temperature. Complete transformation of TNT to DHANT was confirmed by HPLC. The DHANT was used without further purification.

**Protein extraction and fractionation.** *P. pseudoalcaligenes* JS45 cells were grown in BLK with succinate and ammonium chloride. Beginning with cell harvest, all manipulations were carried out at 4 °C. Cells were harvested from mid- to late-exponential phase by centrifugation at 8,000 x g. Pellets were washed twice in phosphate buffer (10 mM, pH 7) and then suspended in 20 ml of the same buffer containing a protease inhibitor cocktail. The cells were disrupted by three passages through a French press at 16,000 psi and the lysate was centrifuged for 20 minutes at 10,000 x g to remove debris and unbroken cells. The crude extract was partitioned into soluble, weakly-membrane associated, and tightly membrane-bound fractions as follows. The crude extract was centrifuged at 200,000 x g. The supernatant contained the soluble protein fraction. The pellet was suspended in 50 mM glycylglycine, 2 M NaBr, 200 mM sucrose buffer with protease inhibitor cocktail and extracted by gentle stirring for 30 minutes. An equal volume of a 50 mM glycylglycine buffer was added and the mixture was centrifuged at 200,000 x g. The supernatant contained the weakly membrane-associated proteins. The pellet, was suspended in 10 mM phosphate buffer pH 7, with Triton X100 0.5% (vol/vol) and protease inhibitor cocktail. Proteins were solubilized by gentle stirring for 60 minutes before centrifugation at 200,000 x g to pellet insoluble materials. The supernatant constituted the membrane-bound protein fraction.

**Enzyme assays.** The reductase activity was monitored spectrophotometrically as the decrease in  $A_{340}$  due to the oxidation of NADPH or NADH in a reaction mixture containing the protein fraction ( $1 \text{ mg ml}^{-1}$ ), NADPH or NADH ( $500 \text{ }\mu\text{M}$ ) and substrate (TNT or NB, at  $100 \text{ }\mu\text{M}$ ) in 1 ml of phosphate buffer ( $10 \text{ mM}$ , pH 7). The mutase activity was assayed by formation of 2-aminophenol as described previously (Davis, Paoli et al. 2000).

## Results and Accomplishments

### Novel metabolic products in TNT transformation pathway.

Transformation of TNT by *Clostridium acetobutylicum*. Previous work completed in the Hughes laboratory identified a hydroxylaminotoluene as an intermediate in the anaerobic transformation of TNT by *C. acetobutylicum*. All efforts to expand the understanding of this transformation pathway demonstrated that further products gained polarity and were extremely oxygen sensitive. No further contaminant transformation products were identified despite attempts using  $^{13}\text{C}$  labeled TNT in conjunction with nuclear magnetic resonance and mass spectrometry. Data in Figure 4 represent an example UV profile of a sample taken from a reactor where *C. acetobutylicum* was exposed to  $^{14}\text{C}$  labeled-TNT. The experiment was allowed to proceed past the point of known metabolites where transformation was verified with the presence of transient hydroxylaminotoluenes and the formation a red colored metabolite. The UV profile showed absorbance that corresponded to aminodinitrotoluenes (ADNTs) which eluted at 10.8 and 11.0 min and the solvent front. Corresponding radioactivity measurements confirmed that all TNT transformation products were associated with ADNTs or with the polar solvent front.

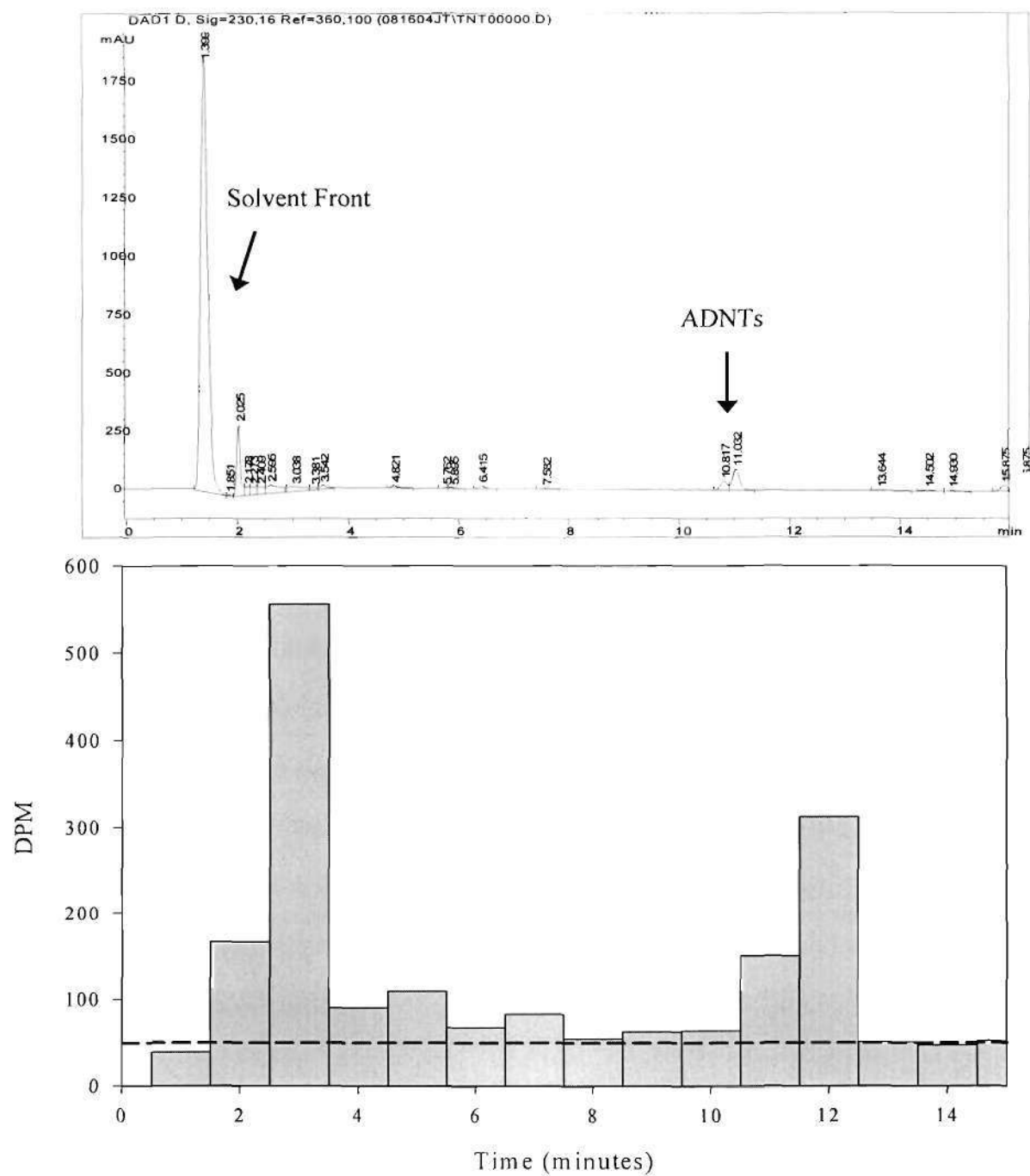


Figure 4. UV profile and radioactivity of effluent of  $^{14}\text{C}$  labeled sample from a reactor where *C. acetobuticum* was exposed to TNT for 14 d. The dotted line represents background radioactivity.



Transformation of TNT by *P. pseudoalcaligenes* JS52. *P. pseudoalcaligenes* JS52, a spontaneous mutant of JS45, was previously reported to produce a polar yellow metabolite from 2,4,6-trinitrotoluene (TNT) after growth on nitrobenzene (NB) (Fiorella and Spain 1997). The metabolite was one of four (Figure 2) distinguished by HPLC retention times and spectra during the initial experiments, but was the only metabolite that required molecular oxygen for its production, during the course of which nitrite was released. The compound was also reported to be persistent once formed in batch cultures. A working hypothesis is that the yellow metabolite is a rearrangement product of 2,4-dihydroxylamino-6-nitrotoluene similar to one detected as an end product in *Clostridium acetobutylicum* transformations of TNT (Hughes, Wang *et al.* 1998). The YM along with the *C. acetobutylicum* product was believed to be a key intermediate in the TNT transformation pathway that leads to unidentified metabolites that no longer display aromatic characteristics.

We combined large experiments in bioreactors with simpler shake flask experiments to determine the time course and optimize the conditions for TNT transformation. Because we believed the yellow metabolite was only produced by NB grown cultures in the presence of NB and TNT we developed a strategy to grow strain JS45 to high cell densities on NB before the addition of TNT for transformation. The toluene-degrading isolate *Acinetobacter* sp. strain F4 will continuously convert diphenylacetylene (not a growth substrate) to a *meta*-ring fission product when a low level of toluene is continuously provided to the culture (Spain, Nishino *et al.* 2003) resulting in the accumulation of high levels of the ring fission product. We attempted to apply a similar strategy to JS45 in order to accumulate the YM from TNT.

*Preliminary experiments.* The work described with JS52 (Fiorella and Spain 1997) involved preparation of the YM in small batch cultures of resting cells. NB and TNT were added to the resting cells with NB in a 4-10 fold excess over TNT. Under those conditions ( $A_{600} = 1$ , TNT = 100-200  $\mu$ M) production of the YM required a 40 min incubation to reach the maximum concentration, and the metabolite was stable for several hours in the culture medium. In an initial experiment, JS45 was grown in a bioreactor in which NB was pumped in at a high enough rate for cell growth, but slowly enough so that NB was never detectable in the culture fluid. When TNT was added, the YM reached the maximum concentration within 25 min, concomitant with the complete disappearance of TNT, after which the concentration declined (Figure 5). A second addition of TNT resulted in a second spike in the yellow metabolite concentration. The preliminary work showed that the yellow metabolite can be further transformed by active cultures, and that transformation of TNT is much more rapid in active cultures than in resting cells.

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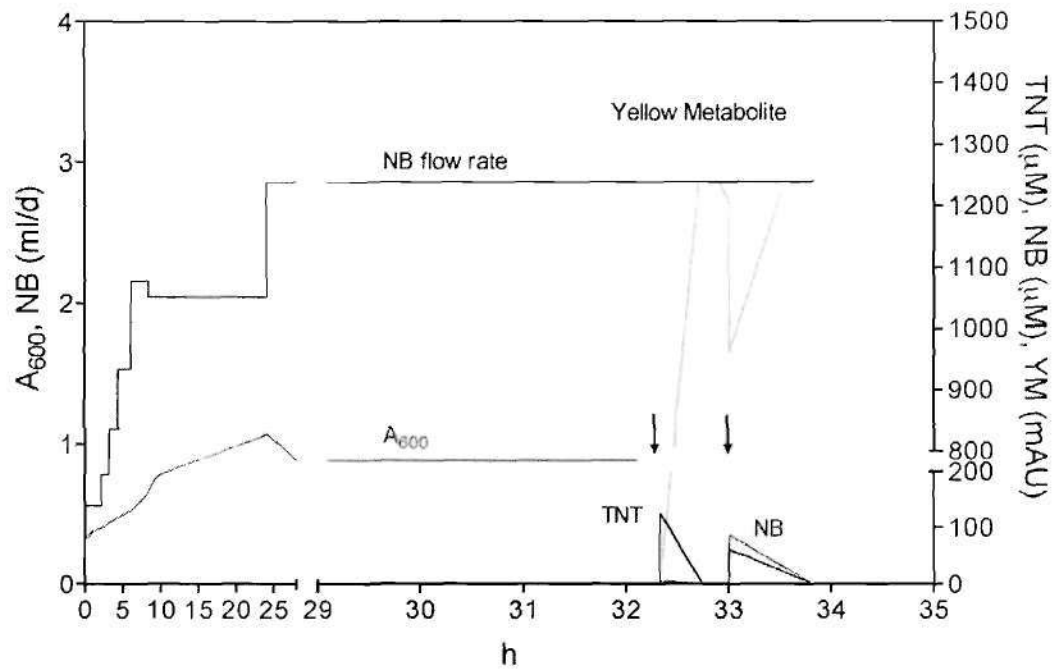


Figure 5. Cultures of *P. pseudoalcaligenes* JS45 actively growing on NB converted TNT (addition indicated by arrows) to the yellow metabolite, which began to disappear after the TNT was exhausted.

*Shake flask experiments.* Studies in shake flasks were designed to optimize the conditions for transformation of TNT. The studies revealed that TNT transformation by resting cells can introduce a lag period during which metabolite accumulation differs from metabolite accumulation in more active cultures. Metabolites that disappeared in active cultures accumulated in shake flask experiments. Shake flask experiments also established a minimum requirement for 2 mol of NB per mol TNT transformed. In practice, higher concentrations of NB are required due to volatilization of NB.

*TNT transformation in bioreactor cultures.* During a TNT transformation experiment in bioreactor culture, 24 metabolites, identified by HPLC retention time and UV-Vis spectra, were detected. The metabolites detected included all the pathway intermediates proposed by Fiorella (Figure 2) (Fiorella and Spain 1997) as well as 2-hydroxylamino-2,6-dinitrotoluene (2HADNT).

*Transformation time courses.* *P. pseudoalcaligenes* JS45 and *E. coli* JS995 were used in time courses for the transformation of unlabelled TNT and 2ADNT as well as  $^{14}\text{C}$ -U-ring-labelled TNT (American Radiolabelled Chemicals, Inc. specific activity = 8 mCi/mmol, radiopurity = 99.14% by HPLC analysis). JS45 was grown on nitrobenzene so that the partially reductive nitrobenzene pathway was induced (Nishino and Spain 1993), and JS995 was grown in LB with 1 mM IPTG and 100  $\mu\text{g/ml}$  of ampicillin. Controls included succinate-grown cells of JS45 and *E. coli* C43(DE), the host strain used to construct JS995. JS995 (Kadiyala, Nadeau *et al.* 2003) contains the genes for nitrobenzene nitroreductase and hydroxylaminobenzene mutase from the nitrobenzene degradation pathway of JS45. When nitrobenzene-grown cells of JS45 were given TNT

or 2ADNT, the yield of YM from 50  $\mu$ M of the initial substrate was 161 and 220 mAU from TNT and 2ADNT, respectively. Uninduced cells did not convert the substrates. When IPTG-induced cells of JS995 were given TNT or 2ADNT, the YM yield was markedly different, with only 23 mAU from TNT, and 330 mAU from 2ADNT. *E. coli* C43(DE) made no YM from either substrate, but approximately 10% of the initial TNT was converted to 2ADNT. Previous experiments showed that nitrobenzene nitroreductase converts TNT to DHANT (Fiorella and Spain 1997); and that hydroxylaminobenzene mutase (HabA) does not convert DHANT to the YM.

Strain JS995 converted TNT mostly to DHANT, and DHANT was not converted to the YM, but disappeared from the culture fluid over several hours in the presence of oxygen. The small amounts of YM made from TNT were probably the result of the formation of 2ADNT by a nonspecific nitroreductase in the *E. coli* C43(DE) host as observed in the control experiments. That the control lacking the genes from JS45 did not convert 2ADNT to the YM while JS995 did, suggests that HabA is involved in the transformation of 2ADNT to the YM. The results further suggest that the wild-type, JS45 converts TNT to the YM only through 2ADNT because the JS995 clone only made the YM from 2ADNT. The yield of YM from TNT and 2ADNT by JS45 further supports the interpretation. One might expect a higher yield of YM from 2ADNT over TNT because some of the TNT is diverted to DHANT. The ratio of the yield of YM from TNT and 2ADNT in JS45 is 0.73 which might approximate the flux of TNT through 2ADNT vs. 0.27 through DHANT in the wild-type bacteria.

The existence of a nitroreductase in *P. pseudoalcaligenes* JS45 that is involved in the conversion of TNT to 2ADNT is also inferred from the above results because nitrobenzene nitroreductase exclusively attacks TNT at the 4-position. Such a nitroreductase is unique in two respects: it preferentially attacks TNT at the 2-position, and 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) is reduced to the amine. No nitroreductase has been characterized to date with those properties.

Time courses with  $^{14}\text{C}$ -labelled TNT and strain JS45 showed that 27% of the initial radioactivity accumulated in the YM peak and 21% accumulated in a second peak with a retention time of 2.1 min by HPLC. Using *E. coli* JS995, the radioactivity initially accumulated in 4HADNT, then shifted to DHANT. As the DHANT disappeared, the radiolabel became dispersed throughout the HPLC fractions, with no distinct peaks of radiolabel. The experiments confirmed that the YM is a significant metabolite of TNT, and also revealed a second less polar metabolite with strong radiolabel. The UV-Vis signal associated with the 2.1 min is not very strong, but the strong radiolabel indicates its importance as a transformation product. An average of 94% of the initial radiolabel was recovered in the HPLC fractions, no mineralization was detected, and less than 3% was associated with the cell mass. Based upon the transformation studies carried out under this project, the TNT degradation pathway used by JS45 has been modified to reflect the major pathway of TNT degradation going through 2ADNT (Figure 6).

6).



*Characterization of the yellow metabolite.* Attempts to purify and concentrate the YM in quantity failed because the YM does not partition into organic solvents (Fiorella and Spain 1997) or form stable derivatives. We were, however; able to produce quantities of YM suitable for LC-MS analysis using an *in vitro* system with purified enzymes. TNT was converted to DHANT by NbzA (Fiorella and Spain 1997). Then partially-purified immobilized HabB (Luckarift, Nadeau *et al.* 2005) was added to the freshly prepared DHANT. The reaction mixture was incubated for 15 minutes at room temperature then briefly centrifuged to remove the immobilized enzyme.

Because HabB rearranges hydroxylamines to *o*-aminophenols (Nadeau, He *et al.* 2000) only a limited number of structures are possible for the YM (Figure 7). LC-MSMS analysis of the reaction mixture showed that the YM yielded a deprotonated molecular mass ion [M-H] at 198 Da (MW, 199 Da). Relevant LC-MS fragmentation patterns (181, 166, 153 and 135 Da) are consistent with an aromatic ring substituted with the following groups: -NO<sub>2</sub>, NHOH and NH<sub>2</sub> (data not shown). The results exclude structure D (Figure 8) and also indicate clearly that the YM is not a ring-fission product. We were unable to isolate the YM in sufficient quantity and purity for NMR analysis because it was unstable.

Although we could not determine the isomeric structure of the YM, it is clear that it is an aromatic compound produced by the rearrangement of DHANT into aminophenol. A similar polar compound (same molecular weight) was produced by a Bamberger rearrangement of DHANT during the degradation of TNT by *Clostridium acetobutylicum* under anaerobic conditions (Hughes, Wang *et al.* 1998). The product produced by *C. acetobutylicum* results from a rearrangement of the 2-position hydroxylamino group to an



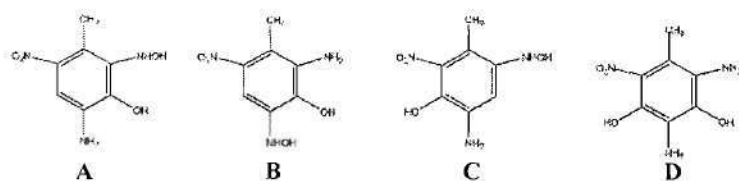


Figure 7. Possible structures resulting from the hydroxylaminobenzene mutase reaction on DHANT. **A**: 2-hydroxylamino-3-hydroxyl-4-amino-6-nitrotoluene; **B**: 2-amino-3-hydroxyl-4-hydroxylamino-6-nitrotoluene; **C**: 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene and **D**: 2,4-diamino-3,5-dihydroxyl-6-nitrotoluene.

amine and the subsequent formation of a hydroxyl group at the 5-position. Furthermore, the products from the transformations of TNT by *P. pseudoalcaligenes* and *C. acetobutylicum* have substantially different HPLC retention times and UV-spectra which supports the conclusion that they are different isomers. HabB, which catalyzes the rearrangement of DHANT to the YM in *P. pseudoalcaligenes*, adds hydroxyl groups at the *ortho*-position exclusively (Nadeau, He et al. 2000; Nadeau, He et al. 2003) whereas an Fe-only hydrogenase has been implicated in the conversion of DHANT to a phenolic metabolite by *C. acetobutylicum* (Watrous, Clark et al. 2003).

### **Characterization of mechanism and enzymes responsible for TNT transformation.**

#### 2,4,6-Trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum*.

The role of hydrogenase in the reduction of TNT by *Clostridium acetobutylicum* was evaluated. An Fe-only hydrogenase was isolated and identified using TNT reduction activity as the selection basis. The formation of hydroxylamino intermediates by the purified enzyme corresponded to expected products for this reaction and saturation kinetics were determined with a  $K_m = 152 \mu\text{M}$ . Comparisons between wild type and a mutant strain lacking the region encoding an alternate Fe-Ni hydrogenase, determined that Fe-Ni hydrogenase activity did not significantly contribute to TNT reduction. Hydrogenase expression levels were altered in various strains, allowing study of the role of the enzyme in TNT reduction rates. The level of hydrogenase activity in a cell system correlated ( $R^2=0.89$ ) with the organism's ability to reduce TNT. A strain that over-expressed the hydrogenase activity resulted in maintained TNT reduction during late growth phases when it is not typically observed in wild type strains. Strains exhibiting under-expression of hydrogenase produced slower TNT rates of reduction correlating with the determined level of expression. The isolated Fe-only hydrogenase is the primary catalyst for reducing TNT nitro substituents to the corresponding hydroxylamines in *C. acetobutylicum* in whole cell systems. A mechanism for the reaction is proposed. Due to the prevalence of hydrogenase in soil microbes, this research may enhance the understanding of nitroaromatic compound transformation by common microbial communities.

The details of this work were published in Applied and Environmental Microbiology (Watrous et al., 2003) as listed in Appendix B and an expanded description

of this work is included in Appendix A1, including tables presenting data supporting conclusions drawn.

Enzymes responsible for transformation of TNT by *P. pseudoalcaligenes* JS45. Fiorella established that NB nitroreductase catalyzes the conversion of TNT to DHANT but not to 4ADNT (Fiorella and Spain 1997). Therefore, some nonspecific but possibly inducible nitroreductase is present in NB-grown cells of JS45 that can catalyze the reduction of 4HADNT to 4ADNT. The significance of another nitroreductase is that 2ADNT has been discovered as another route to the YM. Whether the compound is identical to the YM from DHANT has not been determined. The compounds are indistinguishable by HPLC by retention time and UV-Vis spectra. Both early and late forms are produced. Both intact cells and cell extracts catalyze the transformation. Time course experiments suggest that the route through 2ADNT might be the major route to the YM, but a route that goes through 2ADNT cannot go through DHANT.

Two genes that encode HAB mutases are carried by strain JS45. Both genes have been cloned and expressed in *E. coli*; but only HabA is expressed in JS45 during growth on NB (Davis, Paoli *et al.* 2000). HabB, the protein that is not expressed during growth on NB, however; converts DHANT to the YM, while HabA does not. To clarify some of the anomalies, the JS45 genes that encode nitrobenzene nitroreductase and HabA were cloned into *E. coli* and placed under the control of an IPTG-inducible promoter (Kadiyala, Nadeau *et al.* 2003). Cells and cell extracts of *E. coli* strain JS995 (Figure 8) were incubated with 2ADNT and TNT after induction with IPTG and compared with transformations by nitrobenzene-grown *P. pseudoalcaligenes* JS45. 2ADNT was converted to the YM, whereas

TNT disappeared without the formation of UV-Vis detectable products. During time course experiments with induced cells of JS995, TNT was converted to 4HADNT which then began to disappear, and only traces of YM were identified. A time course with 2ADNT revealed the accumulation and disappearance of the early YM, followed by the appearance of the late YM. An additional unidentified peak (2.4 min peak) not detected during TNT transformation also accumulated and began to disappear. Finally, a similar time course with 4ADNT also showed the accumulation and disappearance of the early YM followed by the appearance of the late YM, and the additional 2.4 min peak accumulated, but did not disappear. The rate of disappearance of 4ADNT was about half that of 2ADNT and TNT. The amount of YM and 2,4 min peak accumulated from 4ADNT was less than half the amounts accumulated from 2ADNT. The time course experiments with JS995 support the interpretation that a pathway through 2ADNT is the major route to the YM. The lack of accumulation of YM from TNT confirms that HabA does not convert DHANT to the YM.

Based on the preliminary results, studies were done to determine the enzyme activities responsible for transformation of TNT, 2ADNT, and DHANT.

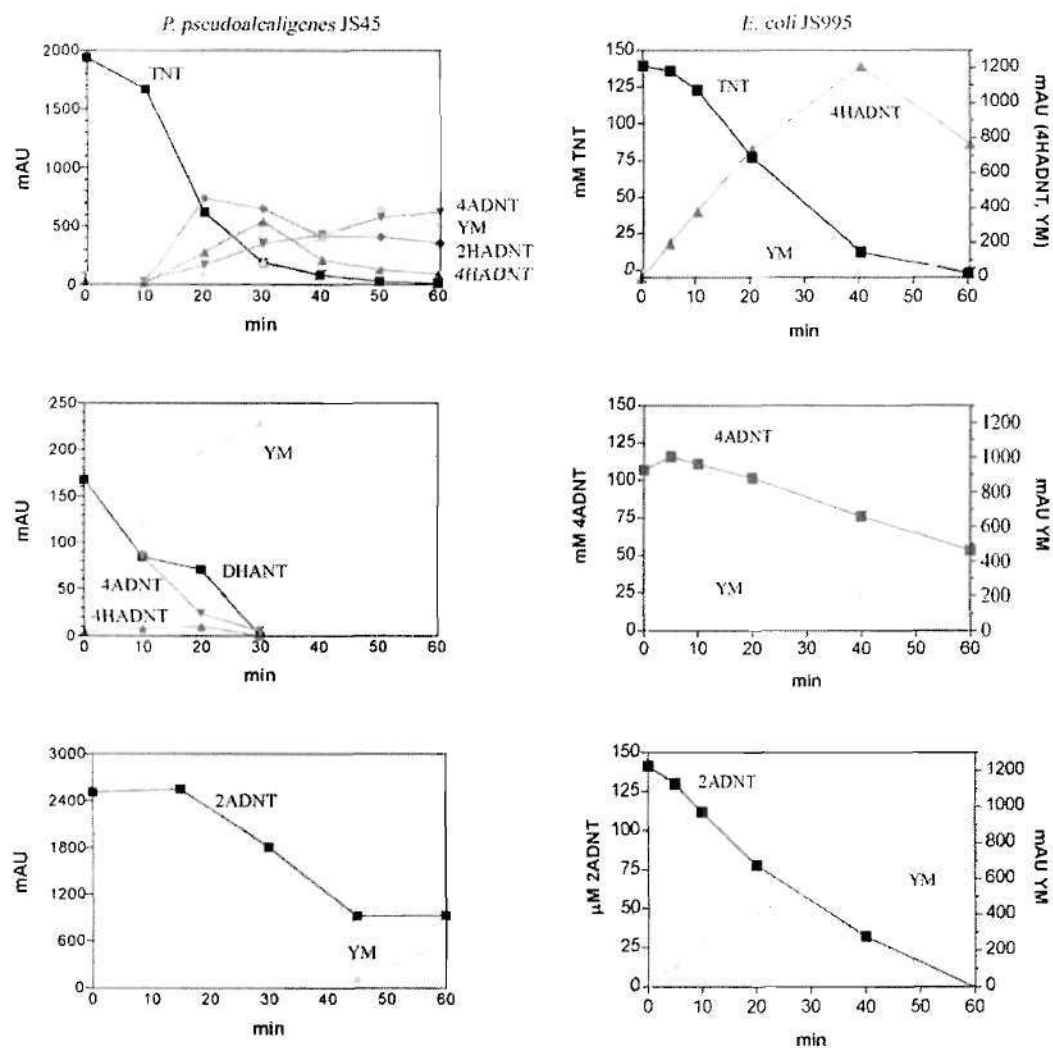


Figure 8. Transformation of A) TNT, B) DHANT, and C) 2ADNT by NB-grown JS45, and transformation of D) TNT, E) 4ADNT, and F) 2ADNT by *E. coli* JS995 after growth in LB and induction by IPTG.

Enzymes involved in the formation of the yellow metabolite. In order to determine whether the hydroxylaminobenzene mutase isoenzymes HabA and/or HabB are involved in the formation of the YM, we tested *E. coli* C43 (DE) strains that express *habA* and *habB* genes under an IPTG inducible promoter, as well as *P. pseudoalcaligenes* JS45 strains  $\Delta A$  and  $\Delta B$  containing deletions of *habA* and *habB*, respectively.

Resting cell experiments were performed with TNT, 2-ADNT and DHANT as substrates. The transformation activities of the succinate-grown JS45 wild type and of JS45  $\Delta B$  lacking *habB* were very similar. Both converted TNT and 2ADNT to the YM, but not DHANT. JS45  $\Delta A$  strain lacking *habA* converted neither TNT nor 2ADNT to the YM. The results indicated that HabA, but not HabB was required for transformation of 2ADNT to the YM.

The *E. coli* strain that expressed HabB converted DHANT but not 2ADNT or TNT to the YM. The *E. coli* strain that expressed HabA converted 2ADNT but not DHANT or TNT to the YM. Strain JS995 converted TNT to DHANT but did not convert DHANT to the YM. JS995 only made the YM from 2ADNT.

Nitrobenzene pathway enzymes from JS45 were partially purified and tested against TNT, 2ADNT, and DHANT. Partially purified nitrobenzene nitroreductase converted TNT to DHANT via 4HADNT as previously reported (Fiorella and Spain 1997). Partially purified HabB converted DHANT, but not 2ADNT to the YM. Partially purified HabA converted neither DHANT nor 2ADNT to the YM.

Previous work indicated that the reduction of TNT to DHANT is catalyzed by NB nitroreductase in cells of *P. pseudoalcaligenes* JS45 and JS52 (Fiorella and Spain 1997).

The current work shows that DHANT can be rearranged to the YM by the hydroxylaminobenzene mutase HabB . However, the rearrangement does not take place *in vivo* because HabB mutase is not expressed by *P. pseudoalcaligenes* JS45 after growth on NB or succinate (Davis, Paoli et al. 2000). Fiorella reported that the YM was formed from DHANT by extracts of NB-grown cells (Fiorella and Spain 1997). The conclusion was based on the results of experiments with crude cell extracts and without a mass balance. We now believe that the cultures used by Fiorella to make the cell extracts likely contained a second nitroreductase that reduced TNT at the 2-nitro position. We infer the lack of induction from the relatively slow TNT transformation rates seen in the earlier work. The results from strain *E. coli* JS995 are consistent with Fiorella's results. TNT was mostly converted to DHANT, and DHANT was not converted to the YM, but disappeared from the culture fluid over several hours in the presence of oxygen. The result was also consistent with radiolabelled experiments, where the radiolabelled DHANT peak disappeared from the *E. coli* JS995 culture leaving only low levels of radiolabeled products over the entire LC chromatogram.

Hydroxylaminobenzene mutase HabA is required to convert 2-ADNT to the YM, but because hydroxylaminobenzene mutases can convert only hydroxylamines to aminophenols (Nadeau, He *et al.* 2003) 2-ADNT cannot be the direct substrate for HabA. 2-ADNT must first be converted to a hydroxylamino compound that can then be rearranged by HabA. The activity that converts 2ADNT into a substrate for HabA is also likely to be a nonspecific activity, being present in both *E. coli* strains and in nitrobenzene-grown as well as succinate-grown JS45 cultures.



Preliminary studies on the TNT nitroreductase. The ability of succinate-grown *P. pseudoalcaligenes* JS45 cells to transform TNT to the YM indicated the participation of a nitroreductase distinct from NB nitroreductase. NB nitroreductase is induced by NB and attacks TNT exclusively at the *para*-position. The nonspecific nitroreductase is part of a constitutive, second pathway in which TNT is converted to 2-ADNT rather than DHANT. The purification of this nitroreductase is currently in progress. Preliminary experiments indicate that the nitroreductase is NADPH-dependent, localized in the soluble protein fraction and has a higher activity with TNT than with NB. Partially purified extracts of succinate-grown JS45 convert radiolabelled TNT to 2HADNT in the presence of NADPH.

Our results suggest that a novel pathway, via 2-ADNT is the predominant TNT transformation pathway in strain JS45. The finding of only traces of radiolabel in DHANT, but significant accumulation of label in the YM supports the findings of the enzyme work. The nitroreductase enzymes involved in the pathway are expressed during growth on NB or succinate and are therefore not induced by NB. The identity of the nitroreductase for reduction of TNT to 2-ADNT has not yet been determined but the nitroreductase is singular in that it attacks TNT at the *ortho* position.

The initial transformation is not specific as indicated by the fact that it took place in both *P. pseudoalcaligenes* and *E. coli*. The reaction was also too rapid to allow detection of the postulated amino-hydroxylamino-nitrotoluene by HPLC.

## **Fate of TNT transformation products in natural systems.**

The reactivity of partially reduced metabolites of 2,4,6-trinitrotoluene in natural systems (*C. acetobutylicum*). The reactivity of partially reduced metabolites of TNT, namely arylhydroxylamines and nitrosoarenes, was evaluated with a simple biological system and with components of soil natural organic matter (NOM). This study was carried out to determine the impact of irreversible binding to soil NOM and biomass, commonly observed during the reductive transformation of polynitroaromatic contamination. The study focused on partially reduced metabolites rather than the completely reduced arylamine metabolites that have already been extensively investigated for their role in binding to soil NOM.

In the simple bioreduction system of *Clostridium acetobutylicum* cell-free extract/molecular hydrogen (electron donor), 10% of the initial  $^{14}\text{C}$  was found bound to solid proteinaceous material following sequential anaerobic/aerobic treatment. A review of the nitroso and hydroxylamino functional group chemistry revealed that the nitroso-thiol reaction was most likely responsible for the reaction with proteins. The introduction of a model thiol, 1-thioglycerol, into an anaerobic mixture of 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 2,4-dihydroxylamino-6-nitrotoluene (DHANT) resulted in the formation of a new product, only when the reaction mixture was exposed to air. The results from the model reaction confirmed that thiols could act as competing nucleophiles for nitroso compounds, which are readily formed from hydroxylamino compounds upon exposure to air.

The reactivity of arylhydroxylamines and nitrosoarenes with standard humic acids was investigated using 4HADNT and nitrosobenzene as model compounds, respectively. Contrary to results reported by others (Achtnich, Pfortner *et al.* 1999), 4HADNT was found to be nonreactive towards humic acid at humic acid concentrations in excess of dissolved organic matter concentrations found in nature. Conversely, nitrosobenzene reacted rapidly with humic acids, with the extent of reaction being highest for humic acids that had a high protein content. Humic acids that were pretreated with a thiol derivatizing agent showed diminished capacity for reaction with nitrosobenzene. Since nitroso intermediates from TNT reduction are difficult to synthesize and are rarely observed in nature due to their high instability, their electrophilic characteristics were evaluated using a molecular modeling approach. Molecular models of potential TNT nitroso intermediates were compared with those of the strongly electrophilic nitrosobenzene. The comparison revealed that 2-nitroso-4-hydroxylamino-6-nitrotoluene was more likely to react similarly to nitrosobenzene than 4-nitroso-2,6-dinitrotoluene.

The details of this work were published in Environmental Science & Technology (Ahmad and Hughes, 2002) as listed in Appendix B and an expanded description of this work is included in Appendix A2, including tables presenting data supporting conclusions drawn.

Fate of *P. pseudoalcaligenes* JS45 transformation products in natural systems. The fate of  $^{14}\text{C}$ -TNT in soil microcosms in the presence of *P. pseudoalcaligenes* JS45 was evaluated. JS45 cultures were grown on succinate or nitrobenzene. The cells were washed in minimal media then suspended in minimal medium with  $^{14}\text{C}$ -spiked TNT (100  $\mu\text{M}$ ) plus the growth substrate (200  $\mu\text{M}$  nitrobenzene or 1 mM succinate). Cultures were divided and freshly collected garden soil was added to one culture (10% wet weight/volume). Cultures were shaken at 200 rpm at 30 °C. Samples were taken at 0, 30, 60, and 120 min. At 60 min, cultures without added soil were again divided and garden soil was added to one of the portions. Samples were centrifuged to remove the soil, and the culture fluid was analysed by HPLC. 10 second fractions were collected and analysed by scintillation counting. Each experiment was run twice.

Figures 9 and 10 show the distribution of radiolabel in aqueous components at each sampling time, with the initial samples on the left. Top rows show cultures without soil, middle rows show cultures with soil, and the bottom row is the culture with soil added after a delay. In all cultures, all the initial radiolabel was in the TNT peak. By 30 min, most of the TNT was converted to 2HADNT, 4HADNT, and 4ADNT. In cultures without soil, most of the radiolabel accumulated in polar products, including the YM as the HADNTs disappeared. 4ADNT was the principal non-polar product that persisted. In cultures with soil, few radiolabelled polar products were detected, and no YM was found in the aqueous phase. Again, 4ADNT was the principal persistent non-polar product.

Following prolonged incubation (3 weeks), roughly half of the radiolabel recovered was found in the acetonitrile-washed soil pellet in cultures with soil added (Table 1). In cultures without soil most of the radiolabel remained in the aqueous fraction.

# NB-grown, Run 1

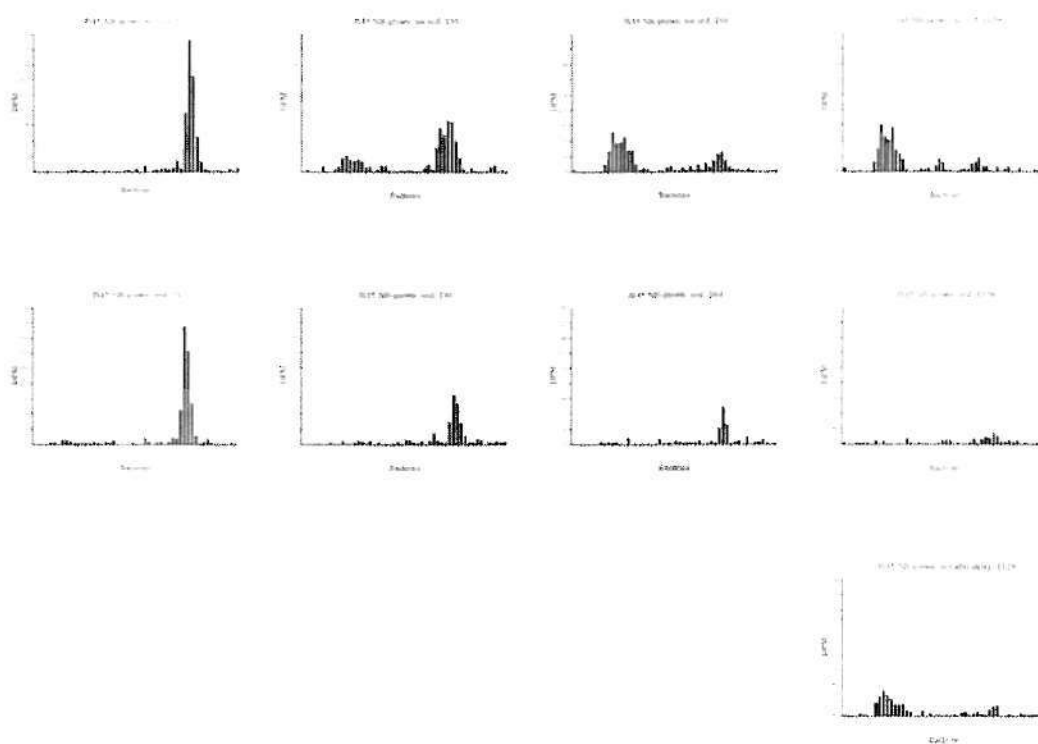


Figure 9. Transformation of  $^{14}\text{C}$ -TNT by nitrobenzene-grown cultures of *P. pseudoalcaligenes* JS45. All figures show DPM plotted on the same scale with 0, 30, 60, and 120 min samples plotted from left to right.

## Succinate-grown, Run 1

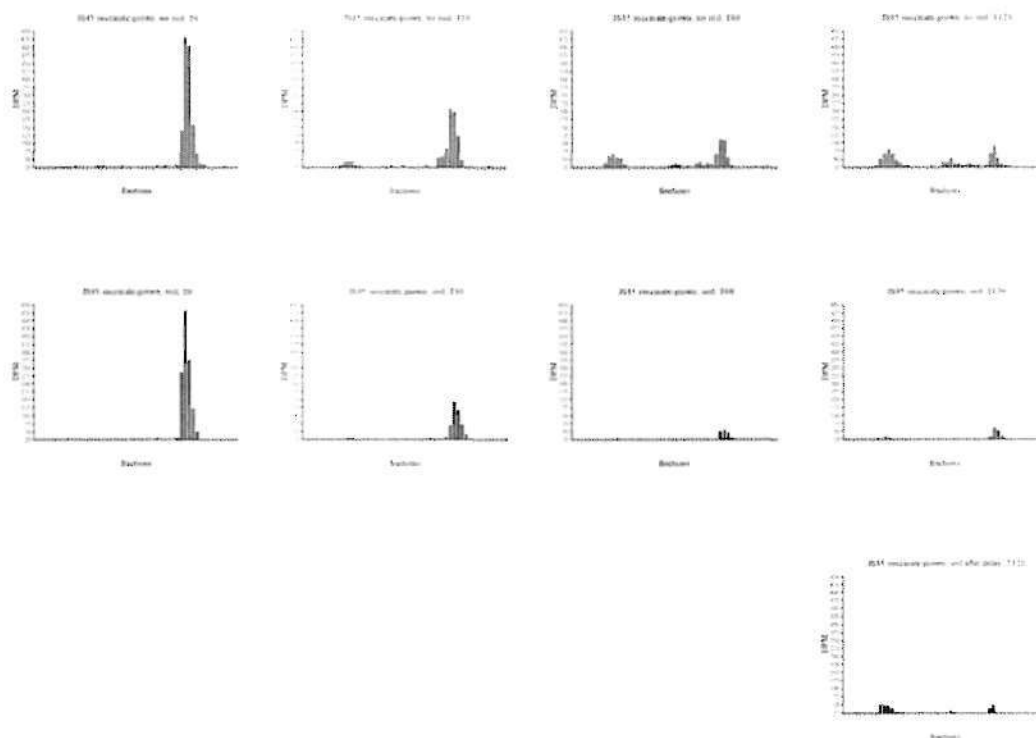


Figure 10. Transformation of  $^{14}\text{C}$ -TNT by succinate-grown cultures of *P. pseudoalcaligenes* JS45. All figures show DPM plotted on the same scale with 0, 30, 60, and 120 min samples plotted from left to right.

Recovery of total radiolabel from cultures with soil was much lower than the total recovered in cultures without soil. Poor recovery was likely due to the inability to sample the larger/heavier soil components in the bottom of the flasks.

The lack of polar metabolites in the aqueous phase of cultures with soil added and the large percentage of radiolabel in the soil, suggests that polar metabolites or precursors to polar metabolites (nitroso- or hydroxylamino- compounds) were bound to the soil in our aerated and stirred systems. Earlier workers found that such compounds became covalently bound to soil components in anaerobic-aerobic composting systems (Daun, Lenke *et al.* 1998; Achtnich, Fernandes *et al.* 1999) and the binding was essentially irreversible (Achtnich, Sieglén *et al.* 1999; Weiß, Geyer *et al.* 2004).

Although both nitrobenzene-grown and succinate-grown cultures rapidly transformed TNT to the same metabolites, nitrobenzene-grown cultures mineralized more TNT than did the succinate-grown cultures (Table 1). The bulk of the mineralization occurred not during the period of rapid TNT transformation, but sometime during the immediate 2 weeks following the initial transformation. The percent of mineralization achieved over a 3 week period is relatively high for bacterial cultures (Weiß, Geyer *et al.* 2004) and the higher mineralization with the nitrobenzene-grown cultures without added soil indicates that some portion of the more polar TNT metabolites are susceptible to the enzymes of the nitrobenzene degradation pathway.

Table 1. Distribution of radiolabel in nitrobenzene- and succinate-grown cultures after 3 weeks incubation as the percent of total radiolabel added.

Fraction	$^{14}\text{CO}_2$				Aqueous	Solids	Total
	Initial	2 weeks	3 weeks	Total			
NB 1	1.1	5.9	1.0	8.0	91.8	0.5	100.3
NB 2	1.1	6.8	1.3	9.2	78.9	1.2	89.3
NB-S 1	0.5	1.8	0.5	2.8	27.7	29.5	60.0
NB-S 2	0.4	2.4	0.5	2.4	20.6	24.0	47.0
NB-DS 1	0.6	5.2	1.4	7.2	40.5	21.5	69.2
NB-DS 2	0.7	4.5	0.4	5.6	30.2	24.1	59.9
Su 1	0.9	1.7	0.4	2.9	66.3	4.0	73.2
Su 2	0.9	2.1	0.5	3.4	65.3	15.2	83.9
Su-S 1	0.7	1.7	0.6	3.0	17.7	23.4	44.0
Su-S 2	0.5	1.8	0.6	2.9	17.3	27.0	47.0
Su-DS 1	0.6	2.2	0.9	4.0	20.5	21.3	45.5
Su-DS 2	0.6	2.6	0.8	4.0	17.1	19.6	40.7

NB, nitrobenzene grown; Su, succinate grown; S, soil added; DS, soil added after 1 h



### **Develop strategies to direct TNT metabolism to ring fission products.**

The project was begun with the assumption that the YM was a ring-fission product, based on its behavior in solvent systems and the UV-Vis spectrum. We now know that the YM is a highly substituted aromatic compound with one of the three structures shown in Figure 7A, 7B, or 7C. Because the YM is not a ring-fission product we redirected our efforts towards determination of how to most effectively use *P. pseudoalcaligenes* JS45 in a field remediation system.

Nitrobenzene is more acutely toxic than TNT and thus is unlikely to be used as a primary growth substrate in TNT remediation systems. However, this project has shown that constitutive enzymes, including the nonspecific nitroreductase(s) and the constitutively expressed HabA will transform TNT almost as well as the fully expressed enzymes of the nitrobenzene-degradation pathway. *P. pseudoalcaligenes* JS45 is a natural environmental isolate and therefore faces few regulatory restrictions on its use in remediation systems. With the idea that the constitutively expressed enzymes of JS45 could be useful in TNT remediation systems *in situ*, we examined alternative carbon sources for cometabolism of TNT by JS45.

*P. pseudoalcaligenes* JS45 was incubated in minimal with i) 50  $\mu$ M succinate as the carbon source and 50  $\mu$ M TNT as the nitrogen source, ii) 50  $\mu$ M TNT as the carbon source and 50  $\mu$ M  $\text{NH}_4\text{Cl}$  as the nitrogen source and iii) 50  $\mu$ M TNT as the sole source of carbon and nitrogen. No growth occurred under these conditions: *P. pseudoalcaligenes* JS45 was unable to use TNT as the sole source of carbon and/or nitrogen. When *P. pseudoalcaligenes* JS45 was grown in the presence of a carbon and nitrogen source (succinate and  $\text{NH}_4\text{Cl}$  or NB), TNT was transformed.

To develop a co-substrate for field implementation, various co-substrates were screened with JS45. Minimal medium + NH<sub>4</sub>Cl was tested with the following carbon sources: glucose 0.5%, succinate 0.5% and molasses 0.1%. Growth and TNT degradation were monitored at different times.

Best growth resulted from molasses as carbon source (Figure 11). Similarly, TNT transformation was most rapid with molasses as carbon source (Figure 12). The molasses-grown cells took less than 24 hours to transform 100 µM TNT, the succinate-grown and NB-grown ones 42 and 52 hours respectively. Interestingly, JS45 was not able to use glucose as carbon source and no TNT transformation was observed in cultures with glucose. Molasses is a cheap and readily available carbon source with demonstrated value for cometabolism of TNT (Manning, Boopathy *et al.* 1995; Widrig, Boopathy *et al.* 1997). Remediation systems based on cometabolism of TNT by JS45 during growth on molasses could be feasible for field application.

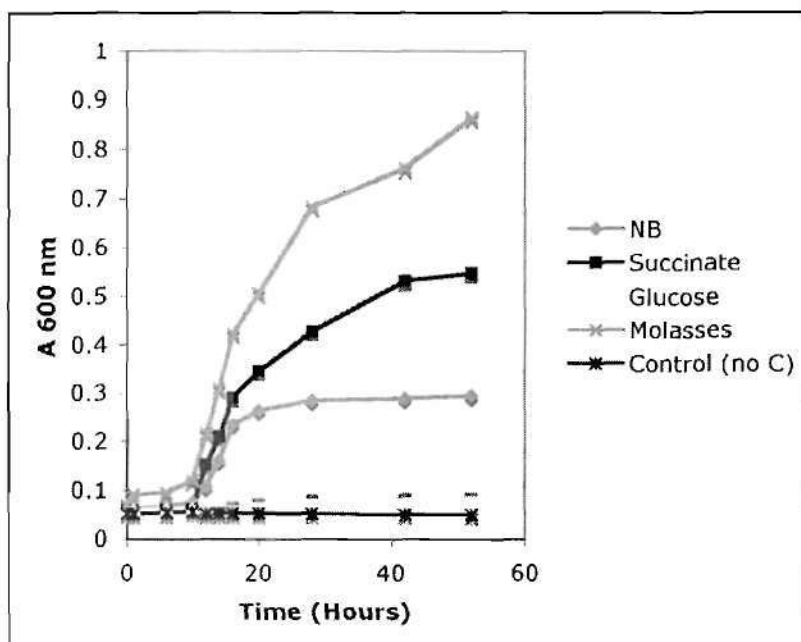


Figure 11. Growth of JS45 with alternate carbon sources.

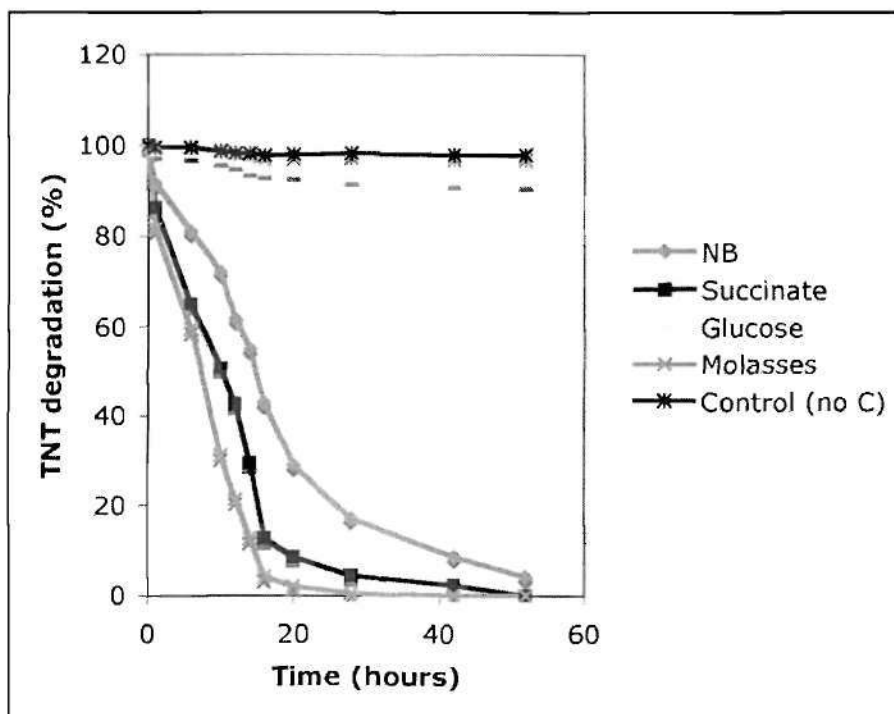


Figure 12. Transformation of TNT by JS45 grown on alternate carbon sources.

## Mineralization of TNT.

Mineralization of TNT using a two-stage anaerobic-aerobic process. Complete mineralization is the desired endpoint of explosives remediation processes because the production of CO<sub>2</sub> represents an unequivocal elimination of the contaminant and all potentially toxic intermediates. Initial studies completed in our laboratory demonstrated that TNT mineralization occurred via combined biotic-abiotic mechanisms during a two-stage anaerobic-aerobic treatment process. Anaerobic incubations of *Clostridium acetobutylicum* with <sup>14</sup>C-TNT produced a reduced hydroxylated metabolite that served as a more favorable starting point for mineralization in subsequent aerobic treatment. Total <sup>14</sup>C recovery during this second stage ranged between 95 and 101% of the initial activity. It was determined that aerobically-maintained reactors with unrestricted growth converted 13.3 to 14.1% of the initial radiolabeled metabolite to <sup>14</sup>CO<sub>2</sub>. This was significantly higher than in the aerobic reactors in which growth was restricted (via autoclaving, mineral omission, or biocide amendments). Production of <sup>14</sup>CO<sub>2</sub> in the restricted-growth reactors ranged from 7.9 to 9.7%, suggesting that an abiotic pathway was responsible for greater than 56% of the observed mineralization yield. Extended anaerobic incubation in place of the aerobic second stage of the dual-step process limited mineralization (1.1 to 1.7% <sup>14</sup>CO<sub>2</sub>) by blocking growth and/or auto-oxidation pathways. The addition of structural analogs to TNT failed to induce mineralization. Inoculation with a consortium enriched on 2,4-dinitrotoluene indicated that mineralization was not tied to the stimulation of specific degrading organisms. The key difference appeared to be the production of a reduced TNT metabolite that proved particularly susceptible to further

transformation and eventual mineralization. This represents the most successful outcome reported to date for an anaerobic-aerobic dual-stage process.

However, results which demonstrated mineralization of TNT were not readily repeatable and therefore were not published in peer-reviewed literature. Subsequent studies showed no significant difference between mineralization observed in active systems and in inactivated controls. Expanded details of studies which demonstrated mineralization and those where mineralization was not present are included in Appendix A3.

Mineralization of TNT by *P. pseudoalcaligenes* JS45. Several short-term experiments with *P. pseudoalcaligenes* JS45 measured mineralization of  $^{14}\text{C}$ -TNT. In all experiments in which incubation times were 3 days or less, very little mineralization was detected (less than 2%). Only during prolonged incubation (discussed above under transformation in natural systems) was significant mineralization detected during a brief period after TNT was no longer detected in the cultures. Mineralization required highly active nitrobenzene-grown cultures and the absence of soil which apparently bound the polar metabolites or their precursors that went on to be mineralized in soil-free systems. Thus, it is unlikely that JS45 could effect much mineralization of TNT in a field remediation system. The strain might, however, be an effective bioremediation agent for TNT-contaminated water in a bioreactor where it could be grown with nitrobenzene under controlled conditions.

## Conclusions

This research examined the biochemical mechanism of TNT transformation in novel aerobic and anaerobic degradation pathways in an effort to advance our understanding of the fundamentals of biological transformation of TNT. Conclusions are summarized for research which investigated reactivity of reduced metabolites of TNT with humic substances and in cell extract systems, and for work which established the enzymes responsible for transformation of TNT in aerobic and anaerobic systems. The unresolved nature of work on mineralization of TNT is described. This fundamental information about biological transformation of TNT will aid in developing strategies that harness the biological transformation of TNT in remediation systems.

The following general conclusions were reached regarding the reactivity of partially reduced metabolites of TNT in a *C. acetobutylicum* cell extract system (a simple bioreduction system):

- Partially reduced metabolites of TNT, namely DHANT and 4-amino-6-hydroxylamino-3-methyl-2-nitrophenol, bind to proteinaceous materials following sequential anaerobic/aerobic treatment.
- The more reduced arylhydroxylamino metabolite of TNT, DHANT, oxidizes upon exposure (to most likely 2-nitroso-4-hydroxylamino-6-nitrotoluene) and readily reacts with model thiols such as 1-thioglycerol.
- Binding between reduced TNT metabolites and proteins occurs most likely by the nitroso-thiol reaction owing to the aerobic requirements of the reaction.

The following conclusions regarding reactive functionalities and substituent effects were drawn from the studies involving the reaction of partially reduced metabolites of nitroaromatics and various IHSS standard humic acids:

- 4HADNT shows no appreciable reactivity towards a standard humic acid (i.e., IHSS peat humic acid standard) under anaerobic conditions. When conditions are made aerobic at pH 7 the 4HADNT precipitates out of solution in the form of 4,4',6,6'-tetranitro-2,2'-azoxytoluene.
- Nitrosobenzene readily reacts with various humic acids.
- The extent of nitrosobenzene binding increases as the protein content of the humic acid increases.
- Thiol derivatization of humic acids adversely affects their reaction with nitrosobenzene with greatest effect shown by the humic acid having the highest protein content.
- Molecular modeling analyses predict that the more reduced nitroso metabolites of TNT such as 2-nitroso-4-hydroxylamino-6-nitrotoluene should be closer in electrophilic character and reactivity to nitrosobenzene than the less reduced 4-nitroso-2,6-dinitrotoluene.

The Fe-only hydrogenase was determined to be the primary enzyme responsible for TNT reduction in *C. acetobutylicum* systems and conclusions regarding the enzyme and mechanism responsible for TNT transformation by *C. acetobutylicum*.

- The Fe-only hydrogenase was primarily considered as the catalyst for TNT reduction as opposed to the Ni-Fe hydrogenase, which is typically associated with hydrogen uptake.



- Characterization of the purified enzyme allowed determination of the N-terminal peptide identical to the 67 Kda Fe-only hydrogenase.
- The enzyme exhibits saturation kinetics with  $K_m$  for TNT of 152  $\mu\text{M}$ .
- Sequence analysis of the hydrogenase gene identified the enzyme as a soluble protein with 4Fe-4S clusters, characterized by the iron-sulfur binding region signatures at amino acid positions 121-132.
- A transmembrane spanning region occurs at amino acid positions 150-200.
- The sequence shows 99% homology to periplasmic (Fe) hydrogenase large subunit of *Desulfovibrio vulgaris*, which may be involved in hydrogen uptake for the reduction of sulfate to hydrogen sulfide in the electron transport chain.

The following conclusions apply to the aerobic degradation of TNT by *P.*

*pseudoalcaligenes* JS45, a soil organism with potential for use in bioremediation systems.

- *P. pseudoalcaligenes* JS45 contains multiple nitroreductases that are specific for nitro-groups substituted at different positions on the benzene ring, and thus direct TNT transformation towards different end products. Metabolic engineering to enhance the activity of selected nitroreductases and/or to silence the expression of nitroreductases that cause unwanted transformations might result in an organism that could be used in a more predictable or robust treatment system.

A constitutively expressed nitroreductase converts TNT to 2ADNT which is subsequently converted to a polar, transient yellow metabolite by the action of the reductase and mutase.

A wide range of aerobic bacteria, including *E. coli*, can catalyze the conversion of TNT to amino derivatives. All of the nitroreductases that have been purified, however, seem to catalyze only the reduction to the hydroxylamino derivatives. It has been a mystery how the conversion of the hydroxylamino compounds to the amines is carried out. In JS45 we have clearly demonstrated the activity of the enzyme that produces amino compounds from TNT. The existence of the enzyme is crucial to avoid accumulation of the much more reactive hydroxylamino compounds. Purification and characterization of the novel enzyme will reveal whether it is widespread and whether its activity is responsible for the often observed accumulation of monoamino derivatives of TNT under aerobic conditions. The advances in understanding from JS45 will allow prediction and enhancement of the activity in a variety of systems. Experiments with radiolabelled TNT reveal that a significant fraction of the TNT can be mineralized while the remainder is converted to polar products or bound to soil. The details of the reactions and the rigorous identification of the yellow metabolite remain to be worked out. Complete understanding of the pathway will provide the basis for enhancing its activity.

- The discovery that *E. coli* strain JS995 containing nitrobenzene nitroreductase and mutase B can extensively transform TNT clearly indicates the efficacy of the two enzymes for elimination of TNT. Additional strain improvement and further

investigation of the process might yield a more effective strain for practical application, particularly in closed systems.

- Nitrobenzene grown cells transform TNT to the yellow metabolite via 2HADNT and DHANT. Nitrobenzene nitroreductase and mutase catalyze the reactions in nitrobenzene grown cells, but not in succinate grown cells.
- JS45 can mineralize TNT when the nitrobenzene degradation pathway is expressed, but mineralization is suppressed by the presence of soil because of binding of polar metabolites of TNT or their precursors. Elucidation of which specific metabolites are mineralized might enable development of strategies to increase the rate and extent of mineralization. Alternatively, the binding to soil might be taken as an effective endpoint.
- JS45 could be used in a bioremediation system to cometabolize TNT with molasses as the primary carbon source. The inclusion of nitrobenzene as an inducer might slightly enhance the extent of mineralization, but not sufficiently to warrant the additional technical difficulties of using a toxic compound in a treatment system. A variety of other systems based on cometabolism of TNT have been used in the past. The advantage of the aerobic system based on JS45 would be that the products are not polyamines and additional bulking of the soil is not required.

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**Appendix A**  
**Supporting Data**

## Appendix A1

### 2,4,6-Trinitrotoluene Reduction by an Fe-only Hydrogenase in *Clostridium acetobutylicum*

## **Appendix A2**

### **Fate of TNT Transformation Products in Natural Systems.**

## **Appendix A3**

### **TNT Mineralization During a Two-Stage Anaerobic-Aerobic Process**

## Appendix B

### List of Technical Publications

Ahmad, F., and Hughes, J. B. 2002. Reactivity of partially reduced arylhydroxylamine and nitrosoarene metabolites of 2,4,6-trinitrotoluene (TNT) toward biomass and humic acids. *Environmental Science & Technology* 36:4370-4381.

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